FURTHERING THE ENZYMATIC DESTRUCTION OF NERVE AGENTS

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ABSTRACT

This study was conducted to investigate the biodegradation potential of neutralized organophosphorus nerve agents: O-ethyl-S-(2-diisopropyl-aminoethyl) methylphosphonothioate (VX), Sarin (GB), Soman (GD), and O-cyclohexyl methylphosphonofluoridate (GF). Removing labile leaving groups from these compounds can be accomplished chemically (e.g. alkali) or enzymatically (by a variety of hydrolases) resulting in stable ionic methylphosphonate esters. Glyphosate utilizing *Burkholderia caryophilli* PG2982 was found to use for growth (as the sole phosphorus sources) low concentrations of ethyl-, isopropyl-, and pinacolyl methylphosphonates (EMPn, IMPn, and PMPn - alkali treatment products of VX, GB, and GD, respectively). Partially purified enzyme was obtained from crude extracts of the *B. caryophilli* PG2982 strain and tested for esterase activity against these phosphonate esters in addition to isobutyl- and cyclohexyl methylphosphonates (iBMPn and CMPn - alkali treatment products of Russian-VX and GF, respectively). Derivatized substrates and products were monitored by GC-FPD. Esterase activities were observed in the order: iBMPn>CMPn>EMPn>IMPn>PMPn. These results demonstrate the potential use of *B. caryophilli* derived enzyme(s) in furthering the destruction of these neurotoxic chemical compounds. This could be an important factor for the US and other nations in attempting to meet the requirements of the 1993 Chemical Weapons Convention agreement to destroy all chemical warfare agents within ten years of ratification (April 2007 for the US).

INTRODUCTION

Neurotoxic chemical warfare (CW) G-type agents: GB, GD, and GF and V-type agents: VX and O-isobutyl S-(2-diethyl-aminoethyl) methylphosphonothioate (Russian-VX or R-VX) are in the stockpiles of the U.S. and former Soviet Union (Chapalamadugu, 1992; Fedorov, 1994; Marrs, 1996; Somani, 1992). The U.S.A. signed the Chemical Weapons Convention agreement (UN, 1992) in 1993 and ratified it on 25 April 1997 that requires the destruction of all CW agents within ten years of ratification. Current chemical decontaminants contain corrosives (e.g. alkali) (Yang, 1992) and incineration has met with community opposition. The U.S. Army is pursuing alternative technologies, such as enzymatic decontamination, that are safe and environmentally friendly (DeFrank, 1993). Degradation of G- and V-type agents can be accomplished using phosphoric triester hydrolase enzymes (e.g. organophosphorus hydrolase [OPH] [Dumas, 1989, 1990] and organophosphorus acid anhydrolase [OPAA] [Cheng, 1993; DeFrank, 1991; Elashvili, 1999]), which - similar to alkali - remove labile leaving moieties resulting in stable ionic methylphosphonate ester products. This enzymatic degradation process for the five selected

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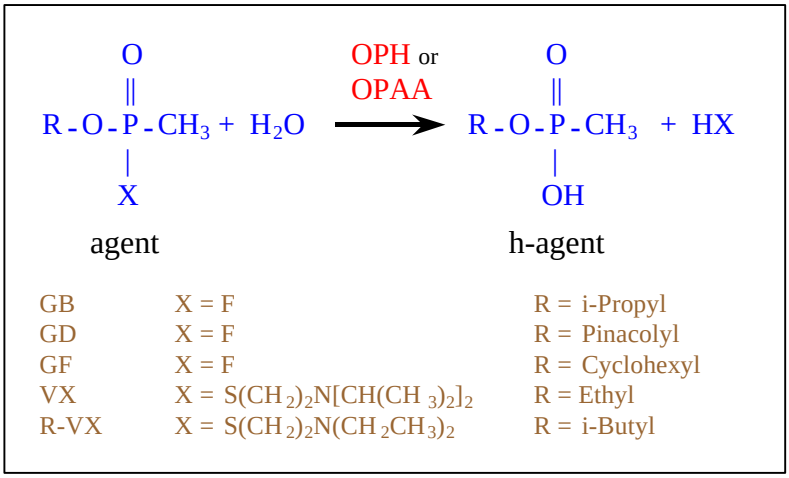
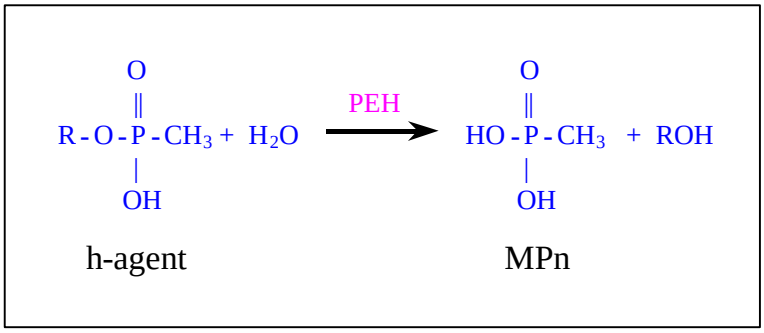
Scheme 1. G- and V-agent hydrolysis by OPH or OPAA.



nerve agents GB, GD, GF, VX, and R-VX would result in h-GB (IMPn), h-GD (PMPn), h-GF (CMPn), h-VX (EMPn), and h-R-VX (iBMPn) products, respectively (Scheme 1).

In search of enzymes that could facilitate further degradation of these chemicals, we screened microorganisms for their ability to grow on methylphosphonate (MPn) and ethyl methylphosphonate (EMPn) as the sole sources of phosphorus. We hoped that these strains might contain enzymes that would degrade all or most phosphonate esters generated during the nerve agent hydrolysis via esterase cleavage resulting in MPn product for the five selected compounds (Scheme 2). We identified two bacterial strains *Burkholderia caryophilli* PG2982 (previously called *Pseudomonas caryophilli* PG2982) and *Pseudomonas testosteroni* that would utilize both MPn and EMPn as the sole sources of phosphorus. The *B. caryophilli* PG2982 strain, which has been known to be capable of utilizing the organophosphorus pesticide, glyphosate, reportedly contains phosphonate monoester hydrolase gene (Dotson, 1996; Moore, 1983; Shinabarger, 1984). It has been reported that the second strain of *P. testosteroni* would utilize alkylphosphonates as the sole sources of phosphorus (Cook, 1978a, 1978b). We investigated these two strains further.

Scheme 2. PEH degradation of hydrolyzed agents.



MATERIALS AND METHODS

Organism and Growth Conditions: The two bacterial strains used in this study were kindly provided by Dr. Braymer (*B. caryophilli* PG2982) and Dr. Alexander (*P. testosteroni*). Unless otherwise indicated, the cultures were routinely grown at 30°C for 40 hours in the new MOPS medium (NMM) adapted from the modified MOPS medium (Elashvili, 1997, 1998) supplemented with 0.3 mM h-GB as the sole phosphorus source. NMM contained (per liter) 8.372 g 3-(*N*-morpholino)propanesulfonic acid (MOPS), 0.717 g of *N*-Tris(hydroxymethyl)methyl glycine (Tricine), 2.92 g of NaCl, 0.51 g of NH_4Cl, 102 mg of $MgCl_2 \cdot 6H_2O$, 10 mg of thiamine, 6 mg of $MgSO_4 \cdot 7H_2O$, 3 mg of nitrilotriacetic acid, 48 mg of K_2SO_4, 1 mg of $MnSO_4 \cdot H_2O$, 2.8 mg of $FeSO_4 \cdot 7H_2O$, 0.1 mg of $CaCl_2 \cdot 2H_2O$, 0.1 mg of $CoCl_2 \cdot 6H_2O$, 0.1 mg of $ZnSO_4 \cdot 7H_2O$, 0.02 mg of H_3BO_3, 0.01 mg of $Na_2MoO_4 \cdot 2H_2O$, 0.01 mg of $CuSO_4$, and 1 g of

glucose, 1 g potassium D-gluconate, 1 g sodium citrate, and the pH was adjusted to 7.0 with KOH prior to sterilization through 0.2µ filter.

To ascertain PEH activities of different bacterial crude extracts, a one-liter Erlenmeyer flask with 300 ml media inoculated with *B. caryophilli* was routinely incubated at 30°C for 40 hours on a recirculatory shaker at 200 rpm. For the enzyme purification, *B. caryophilli* was cultured in four 6-liter flasks at 150 rpm in a total of 10 liters of the medium. The cells were collected by centrifugation at 7,000×g for 30 minutes at room temperature, gently resuspended in a minimum amount of 100 mM Tris buffer, pH 8.0, 100 mM KCl and spun again at 24,000×g for 20 minutes at 4°C. The supernatant was aspirated and the pellet was stored below -85°C. Unless otherwise indicated, subsequent procedures for the enzyme extraction and purification were conducted below 4°C and enzyme fractions were stored below -85°C.

Preparation of hydrolyzed agent substrates: GB, GD, GF, VX, and Russian-VX chemical agents (0.1-0.3 M, CASEARM grade) were hydrolyzed in 1 N NaOH for 2 days and subsequently titrated with HCl to neutral pH. We compared the resulting hydrolyzed agents – h-GB, h-GD, and h-VX – with the corresponding commercially obtained compounds – isopropyl methylphosphonate (IMPn), pinacolyl methylphosphonate (PMPn), and Ethyl methylphosphonate (EMPn) – of the highest available purity. The purity of our hydrolyzed agent preparations was compatible with commercially available corresponding compounds as judged by the analysis of their derivatives on GC-FPD.

Enzyme assays: Enzyme assays were conducted at 30°C with 10 mM substrate in 50 mM 1,3-bis-(tris-[hydroxymethyl]-methylamino)-propane (BTP) buffer, pH 8.5, 1 mM $MnCl_2$, and 2 ìl of enzyme sample in a total volume of 60 ìl. At appropriate time intervals of the reaction, aliquots were withdrawn and added to concentrated sodium hydroxide solution to stop the reaction to make the final NaOH concentration of 1N. Samples were dried, silylated with a mixture of BSTFA+1% TMCS (Pierce) (BSTFA = *N,O*-bis[trimethylsilyl]trifluoroacetamide and TMCS = trimethylchlorosilane) at ca. 110°C for 15 min, diluted with CH_2Cl_2, and analyzed on GC-FPD. The concentrations of the substrates (S_R) in aliquots of the reaction was determined by the formula shown on the right, where S_I is the initial substrate concentration of the reaction, whereas A_S and A_P denote the GC-FPD chromatograms' peak areas of the silylated derivatives of the substrate and product of the aliquot, respectively. The formula is based on the assumption that the ratio of the substrate and product chromatograms' peak area values represent the ratio of their concentrations in the reaction sample. Therefore, the substrate concentration (S_R) in the reaction sample is calculated as the fractional value of the substrate peak area over the combined peak areas of the substrate and the product that is subsequently normalized by the initial concentration of the substrate.

$$S_R = \frac{A_S \cdot S_I}{A_S + A_P}$$

To ascertain enzyme activities in chromatographic effluent samples, 2 mM chromogenic *p*-nitrophenyl phenylphosphonate substrate was used and the room temperature reaction was monitored on a spectrophotometer by measuring the absorbance of liberated *p*-nitrophenol at 405 nm.

Enzyme Extraction: All subsequent enzyme extractions and purifications were conducted at 4°C. Frozen pellets of collected cells were resuspended in 100 mM Tris buffer, pH 8.0, 100 mM KCl, 2mM DTT (3 ml per gram of wet weight). The cells were disrupted by passage through a pre-chilled French Pressure cell (SLM-Aminco) three times at 16,000 psi. Crude cell extracts were obtained after the removal of cellular debris by centrifugation at 37,000×g for 30 min at 4°C. In order to destroy heat labile enzymatic activities in a sample of supernatant, a small portion of the supernatant was boiled for five minutes and precipitates were removed by centrifugation at 13,000×g for 10 minutes.

Enzyme Purification on Ion-Exchange Column: To remove nucleic acids from the crude extract preparation, ten-percent suspension of a cationic polymer Biocryl BPA-1000 (Supelco, Bellefonte, PA) was added to make the final polymer concentration of one percent. After mixing, the extract was incubated on ice for 10 minutes and centrifuged at 37,000×g for 30 minutes. This treatment did not affect the enzyme activity, but it was very effective for nucleic acid removal as judged by the UV (between 200-300 nm) spectral comparisons of pre- and post-treatment samples (data not shown). The supernatant was diluted with 3-fold volume of 4°C chilled 2 mM DTT solution and the sample mix was chromatographed on a DEAE-Sepharose Fast Flow (Amersham/Pharmacia Biotech Inc., Piscataway, NJ) anion-exchange column (25×150 mm). Before the sample application, the column was washed with two column volumes of the 2 M KCl solution and equilibrated with five column volumes of the 20 mM Tris buffer, pH 8.0. After the sample loading, the column was washed with 3.5 column volumes of the 20 mM Tris buffer, pH 8.0, 2 mM DTT, followed by 7 column volumes of the 100 mM Tris buffer, pH 8.16, 100 mM KCl, 2mM DTT to elute loosely bound proteins. The enzyme was eluted with the 100 mM Tris buffer, pH 8.0, 270 mM KCl, 2mM DTT and collected in 20-ml aliquots (Fig. 2). Active fractions (60 ml) were pooled and precipitated with increasing concentrations of ammonium sulfate. Most of the PEH enzymatic activity was recovered in the ammonium sulfate cut between 50-80% (sat.). The resultant pellet was resuspended in the minimal amount of 100 mM Tris buffer, pH 8.0, 100 mM KCl, 2mM DTT and stored frozen below -85°C.

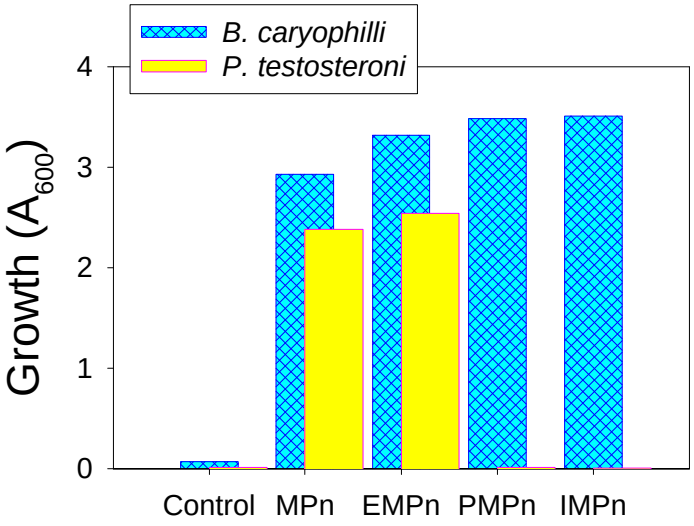


Figure 1. *B. caryophilli* PG2982 has broader phosphonate substrate utilization capability than *P. testosteroni*: Two bacterial strains, *B. caryophilli* PG2982 and *P. testosteroni*, were pregrown on MPn as the sole phosphorus source, washed three times with P-deficient NMM broth and used as inoculums at the seeding A_{600} = 0.010 in NMM broth with 0.3 mM phosphonates as the sole phosphorus source and 2% glucose as the carbon source. After 94 hours growth at 28°C total cell growth was determined turbidimetrically.

Protein Determination: For protein determination, a protein dye binding method (Bradford, 1976) was used, with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Selection of an organism for PEH enzyme purification: The *B. caryophilli* PG2982 strain has been known to be capable of utilizing the organophosphorus pesticide, glyphosate, as the sole phosphorus source and reportedly contains phosphonate monoester hydrolase gene (Dotson, 1996; Moore, 1983; Shinabarger, 1984). Similarly, our tests confirmed that, the *B. caryophilli* PG2982 strain was capable of utilizing the organophosphorus pesticide, glyphosate, as the sole phosphorus source (data not shown). The *P. testosteroni* strain has been reported to utilize alkylphosphonates as the sole phosphorus sources (Cook, 1978a, 1978b). We tested the growth of *B. caryophilli* PG2982 and *P. testosteroni* strains on 0.3

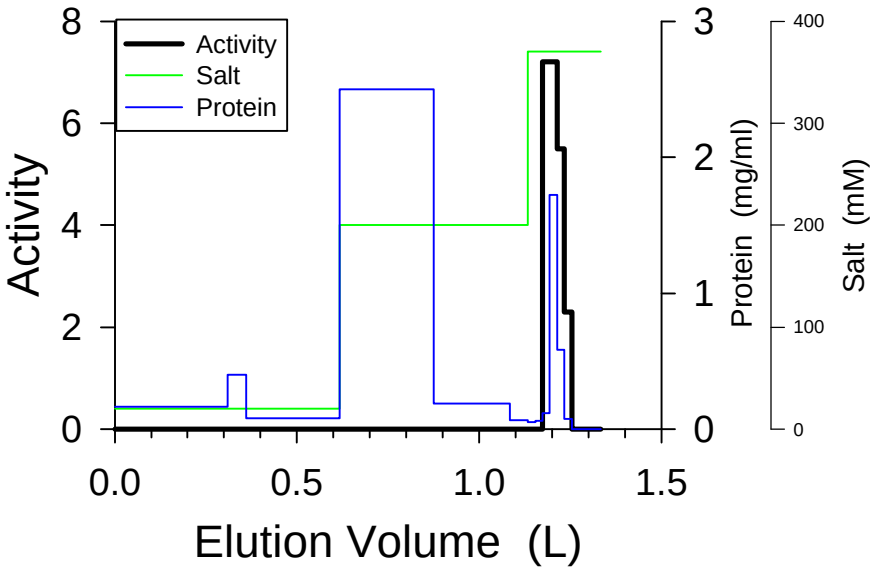


Figure 2. PEH purification on DEAE-Sepharose FF.

mM MPn, EMPn, PMPn, and IMPn as the sole sources of phosphorus (Fig. 1). The latter strain grew only on MPn and EMPn, whereas *B. caryophilli* PG2982 grew on all four phosphonates (Fig.1). No remaining phosphonate substrates were detected in the growth media of the successful growing cultures after the 40-hour incubation period. However, these media contained newly formed inorganic phosphate (data not shown). It should be noted that both the *B. caryophilli* PG2982 and the *P. testosteroni* strains grew on all four phosphonate substrates at 1 mM substrate concentrations as the sole sources of phosphorus, perhaps due to impurities contained in the substrates. However, *P. testosteroni* failed to grow on 0.3 mM concentrations of PMPn and IMPn, while showing robust growth when the phosphorus source was 0.3 mM inorganic phosphate. Since, *B. caryophilli* PG2982 was found to be capable of growth on all the phosphonate ester substrates tested the strain was selected for further investigation.

PEH enzyme purification and characterization: Both the cell-free native crude extract obtained from the *B. caryophilli* PG2982 strain and the denatured (boiled) sample derived from it were tested for the PEH enzymatic activity. The native extract was capable of transforming hydrolyzed nerve agents GB, GD, GF, VX and Russian VX to methylphosphonic acid as judged by the analysis of their silylated derivatives on GC-FPD, whereas the denatured extract had no activity (data not shown). This indicates that the activity was due to a heat-sensitive enzyme in the extract.

A larger scale *B. caryophilli* extract was obtained for purification. After removing nucleic acids and other negatively charged molecules with the cationic polymer, BPA-1000, the enzyme was purified on DEAE-Sepharose Fast Flow columns using KCl step elution. The activity was eluted between 60 ml and 120 ml after the start of 270 mM KCl step (Fig. 2). The active fractions were pooled and concentrated with 50-80% (sat) ammonium sulfate cut.

The partially purified PEH was used to test the enzyme specificity against the selected hydrolyzed agent substrates. The timeline chromatograms of the PEH degradation of two of the substrates, h-GF and h-GD, are shown on Fig 3. As can be seen on the chromatograms on the left panels, the peak denoting the h-GF substrate (the silylated derivative's R_f value ca. 8.24 minutes) decreases with the increase of the enzymatic reaction time, until it finally disappears (after 3 hours), while concomitantly the peak denoting the MPn product (the silylated derivative's R_f value ca. 4.39 minutes) increases (Fig. 3). Similarly, on the right panels, the h-GD substrate (the silylated derivative's R_f value ca. 6.25 minutes) degradation to MPn

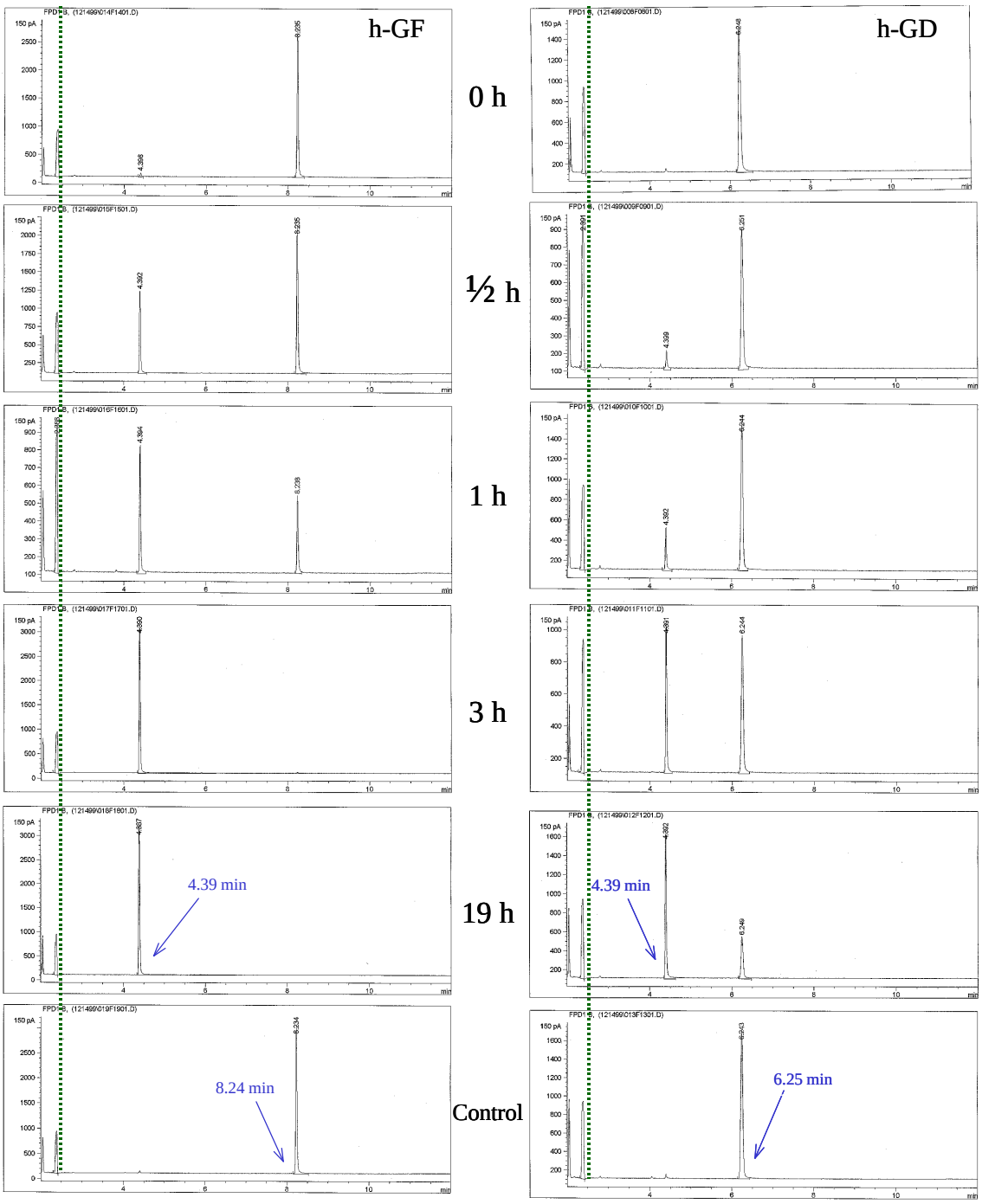


Figure 3. PEH degradation of h-GF and h-GD: The timeline of the GC-FPD chromatograms of the PEH reactions at 30°C with the two (h-GF and h-GD) out of the total of five substrates (ca. 10 mM) and their controls are shown. The 50-80% ammonium sulfate cut of the step gradient purified PEH enzyme constituted ~3.2 % of the total reaction mixture volume. The Control samples represent 19-hour reaction mixtures that contained the buffer used for the enzyme reconstitution instead of the PEH enzyme. The reaction samples were derivatized prior to GC-FPD chromatography. The R_f value of ca. 4.39 minutes of the derivatized enzymatic reaction products of all the five substrates was similar to that of the derivatized MPn standard. The R_f values for derivatized h-GD and h-GF were ca. 6.25 minutes and ca. 8.24 minutes, respectively. (The green dotted lines mark the end of the solvent peaks.)

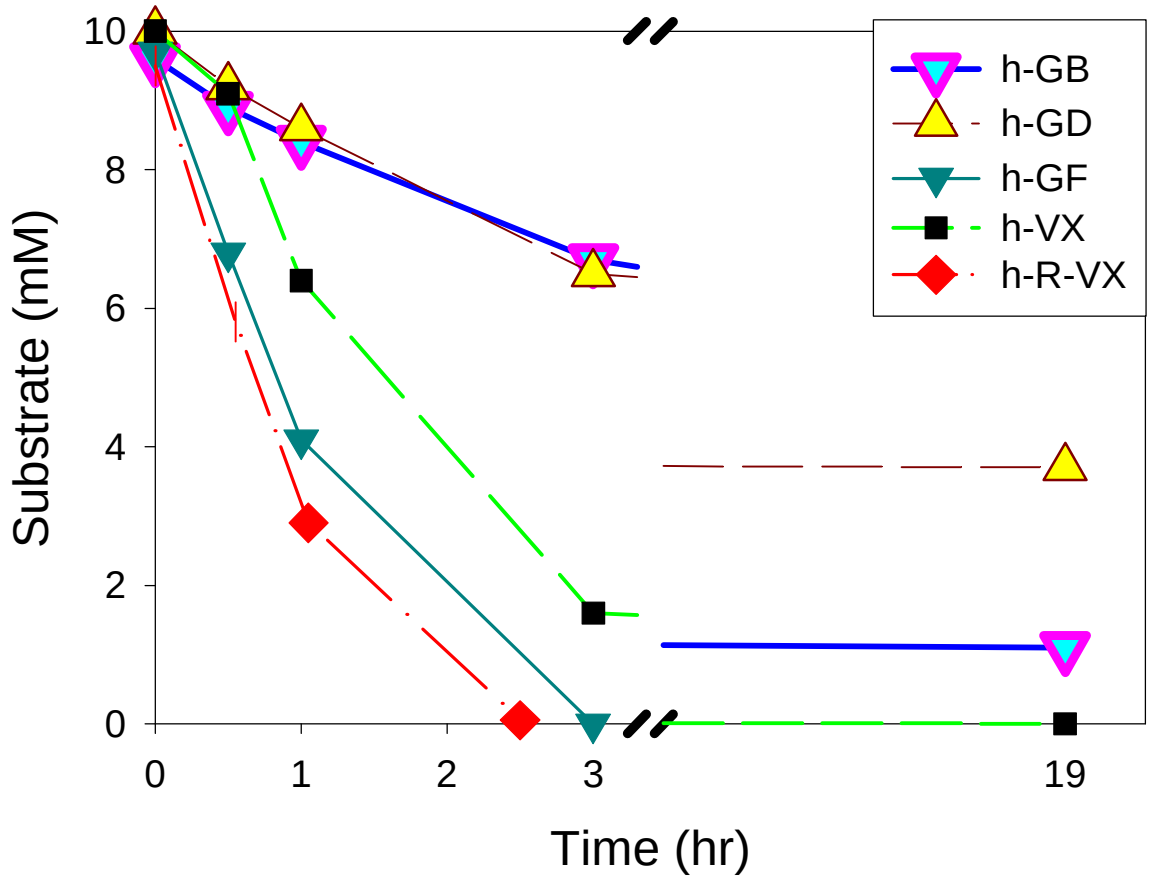


Figure 4. PEH degradation of hydrolyzed agents. The five substrates: h-GB, h-GD, h-GF, h-VX, and h-R-VX were incubated at 30°C with and without PEH enzyme as in Fig. 3. The remaining substrate concentrations were ascertained as outlined in MATERIALS AND METHODS No detectable degradation product was observed for any of the five substrates after 19 hours of incubation in controls without the PEH enzyme.

can be observed, although the enzymatic reaction here proceeds at a slower rate. In contrast, no observable degradation MPn product is visible on the Control chromatogram panels of either of the two substrates (Fig. 3).

The kinetics of the PEH enzymatic degradation of the five selected hydrolyzed nerve agents are shown on Fig.4. In this study the decline of the initial 10 mM h-GB, h-GD, h-GF, h-VX, and h-R-VX substrates are plotted as they are degraded by the PEH enzyme. It demonstrates that the enzyme is most effective against h-R-VX, closely followed by h-GF, both of which were completely degraded within the first three hours of the reaction. Only h-GD and h-GB were not completely degraded after 19 hours of the reaction, the former being the least reactive having 37 percent of the initial substrate still intact, while the latter had only 11 percent of the unaltered original substrate. No detectable degradation product was observed for any of the substrates after 19 hours of incubation in controls without the PEH enzyme.

These experiments demonstrate the effective degradation of the hydrolyzed nerve agents by the PEH enzyme. The order of the PEH activity at 10 mM substrate concentration and BTP bufffer, pH 8.5 was as follows h-R-VX>h-GF>h-VX>h-GB>h-GD (i.e., iBMPn>CMPn>EMPn>IMPn>PMPn).

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